In Vitro Dissolution of Curium Oxide Using a Phagolysosomal Simulant Solvent System

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Detailed study of actinide oxide behavior in alveolar macrophages (AM) *in vitro* is limited because of the short life span of these cells in culture. We created an *in vitro* dissolution system that could mimic the acidic phagolysosomal environment for the actinide and be maintained for an indefinite period so that dissolution of more insoluble materials could be measured. The dissolution system for this investigation, consisting of nine different solutions of HCl and the chelating agent diethylenetriamine pentaacetate (DTPA) in distilled water, is called the phagolysosomal simulant solvent (PSS). In this system, both the pH and the amount of DTPA were varied. We could observe the effect of altering pH within a range of 4.0–6.0 (similar to that of the phagolysosome) and the effect of the molar ratio of DTPA to curium at 1000: 1, 100:1, or 10:1. We chose curium sesquioxide ($^{244}Cm_2O_3$) to validate the PSS for actinide dissolution versus that occurring in AM *in vitro* because it dissolves significantly in less than 1 week. The polydisperse $^{244}Cm_2O_3$ aerosol was generated, collected on filters, resuspended, and added to the PSS solutions and to cultured canine AM. By comparing dissolution in the two systems directly, we hoped to arrive at an optimum PSS for future dissolution studies. PSS and cell culture samples were taken daily for 7 days after exposure and tested for the solubilized curium. The amount of soluble material was determined by ultracentrifugation to separate the insoluble Cm_2O_3 from the soluble curium in the PSS solutions and filtration for the cell-containing material. After separating the soluble and insoluble fractions, the samples were analyzed using alpha liquid scintillation counting. Time-dependent dissolution measurements from the PSS/AM showed that the Cm_2O_3 dissolution was similar for both the PSS solutions and the cultured AM.

Introduction

There have been numerous studies to determine the fate of inhaled materials in the lung. On the basis of these studies, it has been determined that there are two primary mechanisms by which material is cleared from the lung. More soluble material, i.e., material dissolved within approximately 1 day, is solubilized in lung fluids and removed via absorption into the blood. Less soluble, or for the purposes of this discussion, "insoluble," material is removed mechanically. This clearance mechanism involves direct physical movement of material up the mucociliary escalator or phagocytosis by alveolar macrophages (AM), which then transport the material up the mucociliary escalator or move

it through lung parenchyma toward the lung-associated lymph nodes (LALN).

Once inside the phagocytic vacuole of the AM, the inhaled material is combined with a primary lysosome to create a secondary lysosome or phagolysosome. The lysosomal contents consist of numerous enzymes in an acidic environment (*1*) with pH 4–6 (*2*). The phagolysosome works to break down or dissolve foreign material. Phagocytized material is kept in the phagolysosome until it is either solubilized or the cell dies. After the cell dies, any undissolved material is released and phagocytized by another macrophage, in which it again combines with lysosomes, and the dissolution process continues. For insoluble materials such as the actinide oxides and metals that would be phagocytized by the AM but are known to be acid soluble, the acidity of the phagolysosome is responsible for dissolution, and the numerous enzymes present do not contribute significantly to dissolution. Therefore, dissolution of the actinides that occurs in the lungs takes place primarily in the AM phagolysosome.

A primary concern in biokinetic models of the respiratory tract is the dissolution rate and the means by which inhaled material is dissolved in the lung. However, few direct data are currently

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available on actinide oxide dissolution in lung. In the few studies previously reported, material dissolution was measured in simulated lung surfactant or serum (3). This experimental approach is justified for studying the dissolution of more soluble compounds such as uranium yellowcake, in which large fractions can dissolve within hours (depending on chemical form) in surfactant or serum. However, for the relatively insoluble actinide compounds, this dissolution environment is not valid because these materials would be taken up by AM within 24 hr after deposition (4).

For less soluble materials deposited in the lung, most dissolution occurs within the alveolar macrophages. To understand the mechanisms of dissolution and to better predict the behavior of the actinides in the lung, it would be best to study dissolution of a particular compound in AM directly. Unfortunately, lavaged AM can only remain viable in culture for a maximum of approximately 14 days (5). The short viability of AM in culture drastically limits the possibility of performing in vitro AM dissolution studies of more insoluble materials such as plutonium dioxide (PuO_2), which may not demonstrate measurable dissolution in less than 100 days. Short-term studies can be performed in vitro with AM and PuO_2, but the investigator must then assume that the dissolution rate measured in the first few days represents the long-term dissolution rate, which may not be valid (6). Thus, we decided to test a set of solutions that would, in varying degrees, mimic the action of the phagolysosome of the AM for dissolution studies but would allow the studies to continue for an indefinite period. We named this set of solutions the phagolysosomal simulant solvent (PSS) system. Our objective was to directly compare the dissolution with the PSS solutions in vitro and the cultured AM.

We made several basic assumptions developing the PSS solutions that were to serve as surrogates for phagolysosomal dissolution. Although we wanted to identify a solution that was adequate for use in long-term studies, we initially designed a short-term study to compare the dissolution behavior of the PSS system with that of cultured AM for a 1-week period. We chose curium sesquioxide ($^{244}Cm_2O_3$), which has a characteristically high dissolution rate relative to other actinide oxides, to maximize the amount of dissolution achievable in a 7-day period. The composition of the solvent solutions was selected with the proposition that the acidic nature of the phagolysosome is responsible for dissolution of acid-soluble materials. For this reason, we decided to try dissolving the material in an acidic environment with a range of pHs similar to that found in the phagolysosome. By measuring the dissolution rate of $^{244}Cm_2O_3$ in the cell cultures and in the PSS solutions, we hoped to select an optimum solution for use in future, long-term studies with more insoluble compounds such as PuO_2.

Methods

$^{244}Cm_2O_3$ Generation and Exposure

Curium sesquioxide aerosol was freshly prepared using the procedure of Kanapilly and LaBauve (7). Briefly, the ^{244}Cm was separated from its ^{240}Pu daughter isotope by three sequential solvent extractions of curium nitrate with bis(2-ethylhexyl) phosphoric acid in toluene. We added NH_3OH to the purified curium nitrate, filtered the resultant precipitate, and rinsed twice with

distilled water to create a curium hydroxide colloid. We then resuspended the precipitate with a Lovelace nebulizer. The oxide was formed by passing the nebulized $Cm(OH)_3$ droplets through a two-zone furnace with CO_2-free air; the temperature of zone one was 320°C and that of zone two was 1150°C. We then collected the freshly generated polydisperse Cm_2O_3 particles on filters.

To expose the PSS solutions and AM, we suspended $^{244}Cm_2O_3$ in solutions. For a PSS solution, a filter used to collect the particles was sonicated in the solution until the desired specific activity (18.5 kBq/mL) was achieved for a spiking suspension. This was repeated for each PSS solution. We added 1 mL of the spiking suspension to a PSS container with 49 mL of the fresh PSS solution to achieve a final concentration of approximately 0.37 kBq/mL. For exposing the AM cultures, $^{244}Cm_2O_3$ particles were suspended in RPMI 1640, in a manner identical to that described above for the PSS solutions with the same spiking concentration of 18.5 kBq/mL or a particle concentration of 10^5 particles/mL.

Because our preliminary studies indicated that curium suspension concentrations varied with time and that significant amounts of curium were lost to container surfaces, we designed this experiment as a material-balance study. "Dummy" aliquots of the curium spiking suspensions were taken before and after every replicate pair of PSS samples; dummies were also taken after every seven cell cultures spiked. These dummies were then radioassayed for curium content.

Preparation of the PSS Solutions

Aqueous solutions of distilled water and HCl with pH 4.0, 5.0, or 6.0 were prepared. Certain actinide ions form insoluble hydroxides within the pH 4–6 range (8) that can adhere to most container surfaces. Therefore, a chelating agent known to have a high stability constant with the curium relative to the hydroxide compound (8), diethylenetriamine pentaacetate (DTPA), was added to the PSS solutions. The DTPA sequesters curium ions and can compete with the formation of hydroxides in aqueous solutions to prevent the formation of insoluble compounds and reduce losses to vessel surfaces. The DTPA (G. Frederick Smith Chemical Co., Columbus, OH) was put in the PSS solutions at molar ratios of DTPA:curium of 10, 100, and 1000. Surface losses are less likely to occur in cell dissolution systems because numerous proteins capable of binding with metals are available within the cells. We made a total of nine different PSS solutions and studied two replicates, 50 mL each in 125 mL polycarbonate containers (Nalgene Labware, Nalge Company, Rochester, NY), of each PSS type.

Alveolar Macrophage Acquisition and Culture

We obtained alveolar macrophages by whole lung lavage of adult beagles from the ITRI colony using the techniques described by Muggenburg and Mauderly (9). The two dogs used in this study were anesthetized with halothane (Halocarbon Laboratories, Inc., North Augusta, SC), then lavaged with 1000 mL/half lung of sterile isotonic saline solution (Kendall-McGaw Laboratories, Inc., Irvine, CA). The collected cells were washed and centrifuged twice at 135g for 10 min with fresh saline. We determined cell mumbers by light microscopy using a hemo-

cytometer and determined cell viability with the trypan blue dye exclusion technique. Differential cell counts showed that more than 95% of the lavaged cells were macrophages. The cells were diluted to a concentration of 1×10^6 viable cells/mL in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5.0% heat-inactivated fetal calf serum (Gibco) and 0.1% gentamicin sulfate (Sigma Chemical Co., St. Louis, MO).

We placed 6 mL of the cell suspension in each of the 6-cm-diameter sterile polystyrene dishes (Falcon; Becton-Dickinson, Oxnard, CA), and the dishes were incubated for 4 hr at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air to allow the macrophages to attach. Altogether, 56 cell cultures were prepared, 28 for exposure to Cm_2O_3 and 28 controls.

After the incubation period, the medium and unattached cells were poured off and pooled, and an additional 5 mL of fresh culture medium (see above) was added to each culture dish. The final number of cells present in the culture dishes was calculated on the basis of the cell count obtained from the pooled media. The number of unattached cells in the pooled media was averaged over the total number of dishes and subtracted from the original 6×10^6 cells added. We then exposed the cells by adding 1 mL of the Cm_2O_3 particle suspension at 18.5 kBq/mL with a particle concentration of 10^5 particles/mL.

We used two types of control cell cultures for these studies: *a*) cells exposed to fluorescent polystyrene latex (FPL) particles (1.7 μm; Polysciences, Inc., Warrington, PA) at a cell-to-particle ratio similar to that of the AM and the $^{244}Cm_2O_3$ and *b*) cells in medium alone. In addition, two culture dishes containing medium but no cells were spiked with Cm_2O_3 particles to determine the solubility of curium particles in medium alone.

Four of the Cm_2O_3-exposed and two of each of the control cultures were harvested every 24 hr. At each harvest, the cells were scraped from the dishes, counted, and assayed for viability. At the same time, we removed medium and replaced it with fresh medium in those cultures that were not harvested. The spent medium from the cultures containing Cm_2O_3 was kept for curium solubility analysis (see below); the medium from control cultures was discarded. The dishes containing only medium and Cm_2O_3 (no cells) did not have fresh medium replaced during the study but were sampled every 24 hr to measure the time-dependent dissolution. Statistical analyses were done using SAS software. (*10*).

Sampling and Separation of Soluble ^{244}Cm

PSS solutions were sampled immediately after administering the $^{244}Cm_2O_3$ spiking suspensions. Additional samples were taken at 4 and 24 hr after exposure, and then every 24 hr for the 7-day period. The solutions were sampled by sonicating the polycarbonate containers for 1 min, then shaking vigorously by hand to resuspend any particles adhering to the container surfaces. We took three aliquots from each PSS container. Two of the aliquots were analyzed for solubility and the third was analyzed only for total curium concentration by activity.

After counting and assaying viability (see above), the harvested cells and medium were centrifuged (135*g*, 4°C, 10 min) and the supernatant was saved for solubility analysis. The remaining cell pellet was resuspended in 1 mL of 1% sodium dodecyl sulfate (SDS; Biorad, Richmond, CA) solution to lyse the cells. The lysate was analyzed for solubility along with the

supernatant, the spent medium from daily medium exchanges, and the samples from cultures containing curium particles but no cells.

We used two methods to separate the soluble and insoluble fractions: ultracentrifugation for PSS samples and filtration for cell culture samples. For the PSS samples, we used a sucrose gradient (65% by weight) to aid in the separation of soluble and insoluble species in the ultracentrifuge (Beckman TL-100). For a sample of 200 μL total volume, 80 μL of the sucrose gradient was put in the bottom of the centrifuge tube, and 120 μL of the curium-containing sample solution was layered on top. The preparation was centrifuged for 90 min at 400,000*g* and 20°C. After this the sample was fractionated, the top 100 μL was removed by pipette and analyzed for curium α activity, and the remaining liquid and ultracentrifuge tube were analyzed together for curium. Calibration tests with the Beckman TL-100 under these conditions demonstrated that in the top 100 μL the mean percentage of soluble material ($\pm$ SD) was 72.5×0.7 of the total soluble material originally administered. The bottom fraction with the tube contained $99.5 \pm 0.1\%$ of the insoluble material and the remaining soluble amount.

Culture samples were filtered for separation of soluble and insoluble material, then analyzed for alpha activity. To determine the total amount of solubilized curium for a given time-point for the harvested culture, it was necessary to determine the soluble amount in the cell lysate and supernatant at the time of harvesting and add to this the soluble amounts separated when old media was exchanged at earlier times. This additive quantity of soluble material was then divided by the entire amount of material in the culture, determined by adding the separate portions. Small pore-size filters (0.1 μm Nucleopore Filtration Products Corp., Pleasanton, CA, and Millipore Corp., Bedford, MA) were used for this study. Activity measured in the filtrate was considered the soluble fraction, and the remainder on the filter was the insoluble fraction.

Radiochemical Analysis

We used liquid scintillation counting techniques to analyze the separated samples for curium α activity. For samples containing solid materials, we used the techniques of Guilmette and Bay (*11*). Liquid samples were counted by using standard, single-phase α liquid scintillation techniques. Cell-containing material with volume $\geq$ 0.5 mL was wet ashed three times with concentrated HNO_3, dry-ashed at 500°C, dissolved in 3 M HF, then in 2 M HNO_3; the samples were then counted in the presence of a gelling cocktail (Ready Value, Beckman Instruments, Inc., Fullerton, CA). At the end of the study, we also ashed and analyzed the culture dishes to determine whether there had been sizable losses of curium to the walls.

Results and Discussion

Cm_2O_3 Particle Characterization and Exposure

Cascade impactor data taken during aerosol generation revealed the mean activity median aerodynamic resistance diameter ($\pm$ SD) for the particles was 0.91 ± 0.08 μm with a mean geometric standard deviation of 1.8. From this data, the mean mass median diameter (MMD $\pm$ SD) was 0.23 ± 0.03 μm,
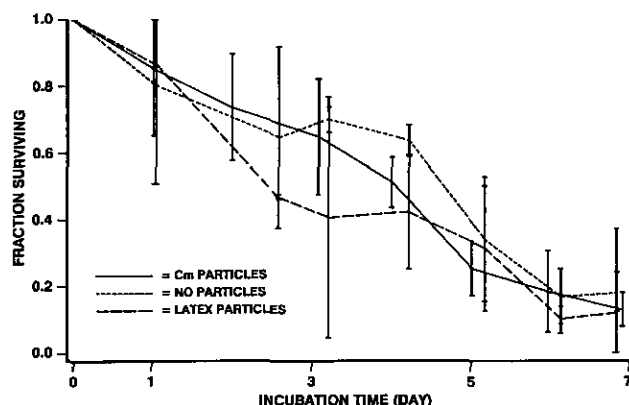
FIGURE 1. Cell survival. Fraction of adhered cells (mean ± SEM) surviving from time of exposure to end of study for the three cell populations (control, with Cm_2O_3 particles, with fluorescent polystyrene latex particles).
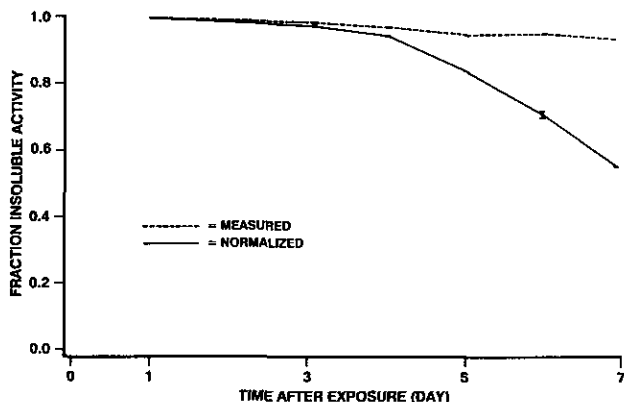

FIGURE 2. Dissolution of $^{244}Cm_2O_3$ in cell cultures during study as a function of time from exposure including the mean insoluble fraction (± SEM). Dissolution for normalized cultures, defined as the soluble fraction divided by the average fraction of cells surviving at midpoint between harvest times.

assuming particle density of 8 g/cm³ as determined previously for Cm_2O_3 at the Inhalation Toxicology Research Institute (12). Spiking suspensions for the PSS solutions had particle concentrations of roughly 10^5 particles/mL, with an overall PSS solution concentration of 2×10^3 particles/mL. In the cell spiking solutions, the particle concentration, based on the MMD and total activity, was 8×10^4 particles/mL with a final cell-to-particle ratio of 60.

Cell Viability

Time-dependent cell survival for both the control and the curium-treated cell populations is shown in Figure 1. The survival trends for the three cell populations were the same within the variability of the data. The mean cell survival (± SD) at the end of the 7-day period was 12.2 ± 12.5% for the controls with FPL particles, 18.2 ± 4.0% for the controls without particles, and 13.0 ± 5.2% for the curium-treated cells. Thus, the presence of ^{244}Cm at the level used in this study did not affect cell survival.

Dissolution of $^{244}Cm_2O_3$ in Alveolar Macrophages

First, uptake of the Cm_2O_3 by the cells was determined. This was done by evaluating the activity associated with the cell lysates at the 24-hr harvest. In relation to the total activity, the mean percent activity (± SD) that was in cells or cell associated at 24 hr was 92.3 ± 0.8%. For the same cell cultures, about 3% of the activity adhered to dish surfaces. This demonstrates Cm_2O_3 uptake to be fairly high in the cell population, which was expected. For this reason, analysis of dissolution in AM was done assuming complete uptake.

Solubility data revealed that little dissolution occurred during the 7-day period in AM. This result was not expected because inhalation studies with Cm_2O_3 in dogs showed that 22% of the initial lung burden had already translocated to liver, bone, and other extrapulmonary tissues in 8 days (12). The in vitro dissolution data from this study were then reevaluated by normalizing the data for the number of viable cells available to dissolve the curium. To do this for a specific harvest time, the insoluble fraction was divided by the fraction of viable cells present at that time. The fraction of viable cells present was determined by dividing the mean number of cells surviving at midpoint between harvest times by the mean attached cell number calculated for all culture dishes after the 4-hr incubation. This worked to "normalize" the insoluble fraction to the viable cell number. We used this method because it accounted for the decreasing number of cells available for dissolution as the study continued. Figure 2 shows the results of this analysis along with the original dissolution curve. This analysis demonstrates that approximately 45% dissolved in 7 days, a result that is not in close agreement with the in vivo data (12).

This disparity between in vitro and in vivo results may be due to several factors. First, the method used here of harvesting, lysing, and analyzing cells disrupted the cells to measure the soluble fraction. This technique hastened the release of soluble material. The in vivo data (12) show the amount of material translocated from the lung, but the solubility of the material remaining in the lung is unknown. Thus, the in vivo data do not account for soluble material in the lung that may be in cells or otherwise bound in pulmonary tissues. Also, in our normalization it was assumed that insoluble material released from dead cells would not be rephagocytized by other macrophages in the culture. This assumption inflates the calculated soluble fraction because some rephagocytosis may occur with the remaining viable cells. Therefore, we concluded that the apparent differences between the results of the previous in vivo study (12) and those of the present study were not as significant as they first seemed.

Based on the results of the dissolution analysis of media from cultures without cells, there was no significant change in the activity in the soluble fraction over the 7-day period. Throughout the course of the study, the soluble activity (± SD) averaged 5.6 ± 2.9% of the total activity. In analyzing these culture dishes, we found that > 60% of the curium adhered to the polystyrene surfaces of the culture dishes. This was not observed with the cell-containing cultures. Thus, the presence of the cells appears to have modified the behavior of Cm_2O_3 within the cultures by preventing the particles from adhering to surfaces.

Dissolution of ^{244}Cm$_2$O$_3$ in the PSS Solutions

Our preliminary studies proved that losses of actinides in solutions with pHs like those of the PSS solutions are common. Oxide particles adhered to surfaces along with insoluble hydroxide compounds formed by the actinides shortly after dissolution in aqueous solutions. This was seen earlier for dissolution of Cm$_2$O$_3$ without chelator where losses increased with time. In our previous investigations, the soluble compound curium-DTPA did not demonstrate measurable losses in the range of pH 4–6, indicating that the hydroxide compounds adhering to container surfaces are probably insoluble forms.

Because we suspected that some surface adherence may occur during this study, PSS solution sample concentrations were analyzed to detect any time-dependent decreases in activity that would indicate surface losses of curium in the PSS containers. This was carried out by determining the activity of samples that were taken but not separated into fractions. We discovered losses for total curium concentration versus time for the PSS solutions with the lowest DTPA to curium ratio, 10:1. Increased losses were associated with increasing solution pH and decreasing DTPA: curium ratio.

Because DTPA would not be expected to complex Cm$_2$O$_3$ particles, the various decreases in curium concentration probably represent solubilized curium that formed an insoluble hydroxide and adhered to surfaces when not complexed by the DTPA. Also, pH plays a role in determining container surface adherence at these low DTPA concentrations by affecting the hydroxide formation, which increases with increasing pH (5). For the PSS solutions that experienced measurable losses, the time-dependent decrease in total curium activity in the solutions was 0.45± 0.20 kBq/day for pH 4, 0.63 ±0.23 kBq/day for pH 5, and 0.82 ±0.24 kBq/day for pH 6. Each of these solutions started with approximately 18.5 kBq. Losses were negligible at DTPA:curium ratio of 1000:1 and 100:1.

Analysis of the time-related amounts of curium dissolved revealed definite trends in dissolution. The differences appear to relate to both pH and DTPA:curium molar ratio. Figure 3A–C demonstrates the effect of pH on the dissolution properties of the solutions at constant DTPA:curium ratios. It is evident that, for a given DTPA:curium ratio, dissolution rates increased with decreasing pH, as expected for this acid-soluble form of curium.

The Cm$_2$O$_3$ dissolution as a function of DTPA:curium ratio at constant pH is shown in Figure 4A–C. The results show that there was less observable dissolution at a DTPA:curium ratio of 10 than at ratios of either 100 or 1000, but that there was little difference in dissolution between the latter ratios. This was the case for all three pH values tested. Thus, we postulated that the chelating agent, DTPA, may affect dissolution behavior in these PSS solutions in two ways. First, DTPA may participate directly in the dissolution process by acting on the curium atoms on the surface of the Cm$_2$O$_3$ particles, thus accelerating their removal into the aqueous phase. Second, DTPA may form stable, soluble complexes with curium that has already dissolved; this chemical form may have less of a tendency to adhere to the container surfaces, thus reducing wall losses. The data from this study do not allow us to differentiate clearly between the two mechanisms; however, the latter may be more important, based on the stronger pH dependence of the solubility than on DTPA concentration and the lack of difference seen at DTPA:curium ratios of 100 and 1000.
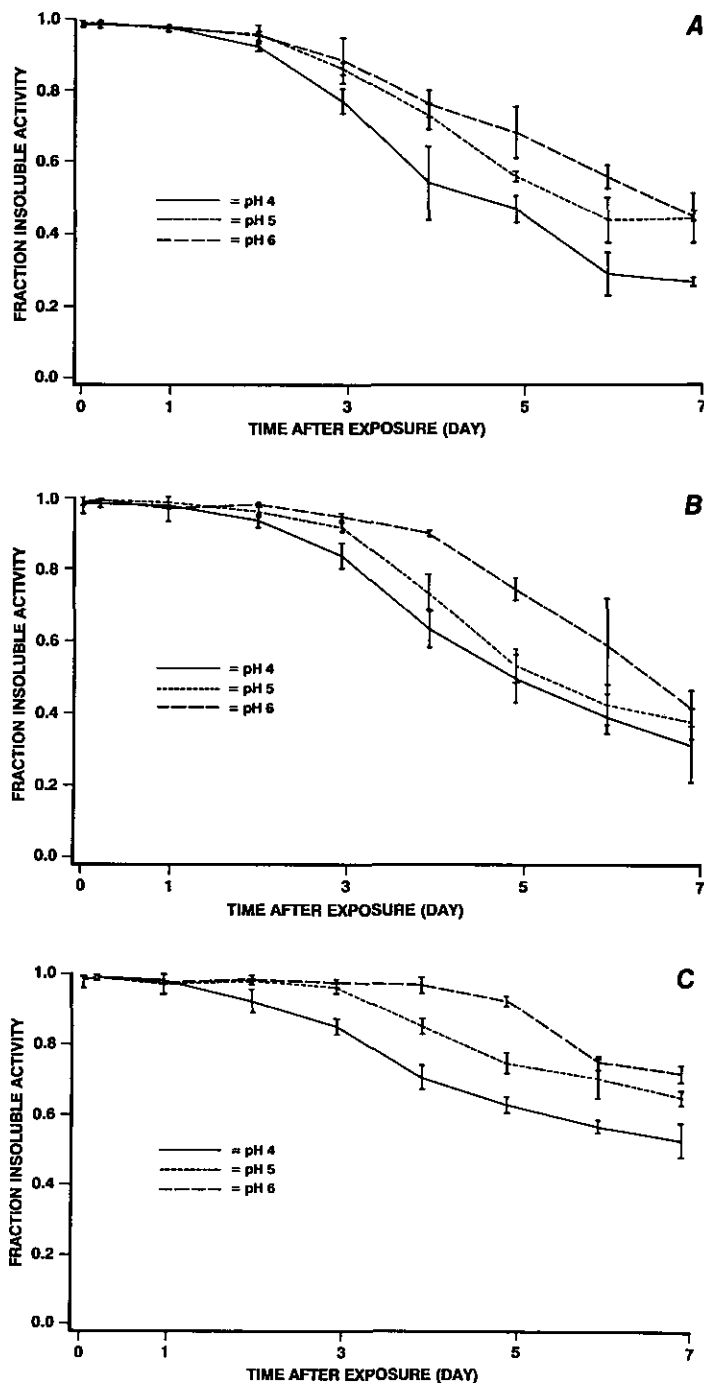

FIGURE 3.   Effect of pH on time-dependent dissolution of ^{244}Cm$_2$O$_3$ in phagolysosomal simulant solvent solution system with the mean undissolved fraction (± SEM) for constant diethylenetriamine pentaacetate to curium ratios: (A) 1000: 1, (B) 100: 1, (C) 10: 1.

Comparison of the cell-associated dissolution of Cm$_2$O$_3$ and dissolution observed with PSS solutions shows several similarities in curve shape and in total amount of curium dissolved in 7 days. It is interesting to speculate on the shape of the curium dissolution curves, which were similar for both the cell-containing system and the PSS solvent systems. In all cases, there
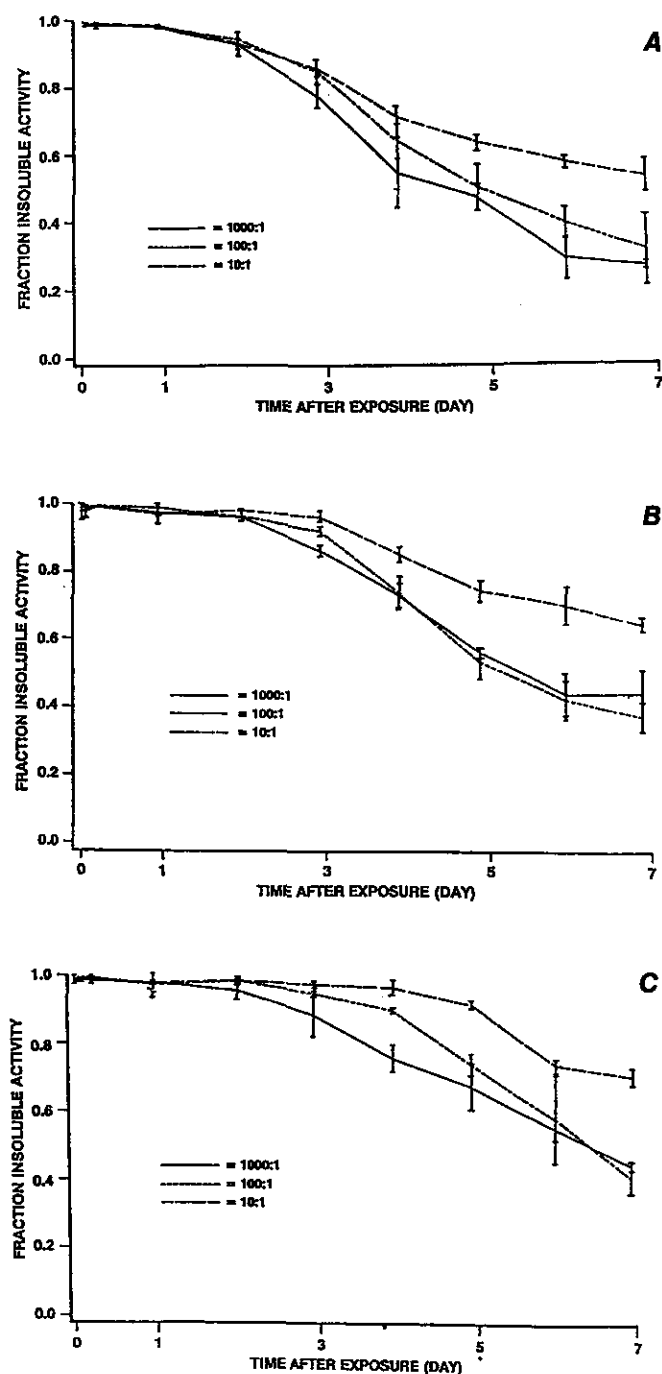
face of the particle was less than for those atoms contained at some depth within the particle. Second, it is possible that α-radiation damage to the particles from curium decay could produce damage to the crystal lattice, resulting in fragmentation of the particles and an increase in specific surface area. This phenomenon has been described for the high-specific activity isotope ^{238}Pu by Diel and Mewhinney (13). We plan to do future studies with monodisperse Cm_2O_3 particles to determine whether fragmentation plays a role in the dissolution of curium aerosols.

Conclusion

The purpose of this study was to identify an effective *in vitro* surrogate for the alveolar macrophage for indefinite-length dissolution studies of acid-soluble metal and oxide aerosol particles. This was accomplished by testing a series of aqueous solutions with slightly acidic pHs and the complexing agent, DTPA. Several notable observations resulted from this study. Apparently, a DTPA:curium ratio of 100 is as effective at complexing the soluble curium as a ratio of 1000:1. At a ratio of 10:1 there is some loss of curium in the samples; we assumed that some of the solubilized curium did not complex with the DTPA, formed an insoluble hydroxide, and adhered to the container surface. On the basis of our results, we assumed that the DTPA did not measurably affect the dissolution rate but that it did play an important role in keeping the solubilized curium in solution for measurement. Lower pH appeared to enhance dissolution by increasing the formation of Cm^{3+} directly. Because the study of Cm_2O_3 dissolution using AM produced ambiguous results, it is too early to identify an optimum PSS composition at this time. Studies are currently being conducted to determine the validity of using AM for *in vitro* dissolution of the actinides.

FIGURE 4. Effect of diethylenetriamine pentaacetate to curium ratio in phagolysosomal simlant solvent solution with the mean undissolved fraction ($\pm$ SEM) for constant pH: (A) pH 4, (B) pH 5, (C) pH 6.

was little dissolved curium observed for the first 3 days after the beginning of the study. Subsequently, the dissolution rate increased significantly, giving the curve a concave-downward appearance. There are at least two mechanisms that can be proposed to explain this behavior. First, it is possible that the curium particles might have a heterogeneous physicochemical composition such that the solubility of the Cm_2O_3 molecules on the sur-

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